Studies on the amino acid incorporating system of soybean seeds and seedlings(Dissertation_全文)

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CONTENTS

ABBREVIATIONS

ATP, adenosine triphosphate

ATPase, adenosine triphosphatase

CMP, cytidine monophosphate

dCMP, deoxy-CMP

CMPase and dCMPase, nucleotidase activities when CMP and dCMP were used as the substrates, respectively

CTP, cytidine triphosphate

DNPP, bis-p-nitrophenyl phosphate

EDTA, ethylenediaminetetraacetic acid

FDP, fructose-1, 6-diphosphate

GMP, guanosine monophosphate

dGMP, deoxy-GMP

GTP, guanosine triphosphate

mRNA, messenger RNA

PDase, phosphodiesterase

pNPG, p-nitrophenyl phosphate

RNA, ribonucleic acid

rRNA, ribosomal RNA

RNase, ribonuclease

sRNA, soluble RNA

TCA, trichloroacetic acid

tRNA, transfer RNA

UMP, uridine monophosphate

XTPase, nucleoside triphosphatase

0-coty, 2-coty, 4-coty, etc., cotyledons from the seeds soaked overnight in water and from 2 day-, 4 day-old seedlings, etc.

3-hypo, hypocotyls prepared from 3 day-old seedlings

CHAPTER I

INTRODUCTION

An outline of the mechanism of protein synthesis has been demonstrated from many studies *in vitro* with ribosomal systems of bacterial (e.g. *E. coli*) and animal (e.g. rat liver, reticulocytes) origins[1-3]. And also it has been believed that the mechanism of protein synthesis in plants is fundamentally the same as in microorganisms and animals from the studies with the ribosomal systems from pea seedlings[4-6], maize kernels[7-9], wheat endosperm[10-13], peanut cotyledons[11, 12] and castor bean seedlings[14].

On the other hand, the germination of seeds has been studied from various point of view. For example, it is well known that the enzyme activities in seeds increase with the progress of germination (pea[15], barley[16]), which may be due to the activation of protein synthesizing system in seeds with germination.

In the present studies, with refference to the above view points, it was attempted to study the amino acid incorporating system *in vitro* of soybean seeds and seedlings to elucidate the activation of protein synthesizing system in soybean seeds with germination from various aspects of elements (ribosomes, transfer RNAs, messenger or template RNAs, polysomes, enzymes involved in protein synthesis, etc.) involved in the system. It was also attempted to study whether the elements in soybean seeds have its own characteristic features. The details of these experimental results will be described in the following chapters.

CHAPTER II

ENZYME ACTIVITIES ASSOCIATED WITH RIBOSOMES FROM SOYBEAN SEEDLINGS[a)]

1. INTRODUCTION

A large number of enzymes have been found in association with ribosomes from various sources; microorganisms, animal tissues and pea seedlings[17)]. The nature of ribosomal enzymes has been studied in detail with E. coli[18-21)]. The enzymes which were found in ribosomes were also found in cytoplasmic solution, and the relation between these enzymes and ribosomal functions is not clear. The enzymes in the ribosome fraction may be (a) newly synthesized enzymes which were not released from the ribosomes yet, (b) soluble enzymes adsorbed on the ribosome surface which were solubilized on disruption of the cells, or (c) enzymes that were the structural components of ribosomes. Or the ribosomal enzymes may exist as the mixture of these three status.

The auther tried to examine some differences between enzyme activities of the ribosomes from resting-tissues and those from working-tissues. This chapter deals with the enzyme activities found in the ribosomes from the cotyledons of soybeans which were soaked overnight in water (resting-cotyledon ribosomes) and ribosomes from cotyledons and hypocotyls of soybean seedlings (working-cotyledon ribosomes and hypocotyl ribosomes).

2. MATERIALS AND METHODS

Preparation of Ribosomes

Soybeans were germinated in vermiculite at 25°in the dark

for 5 days. Hypocotyls and cotyledons were separated from the seedlings, and the ribosomes (hypocotyl ribosomes and working-cotyledon ribosomes) were prepared. Resting-cotyledon ribosomes were prepared from soybeans which were soaked overnight in water at 4°and from which the germs were eliminated. The materials were ground with 0.5 M sucrose solution. The homogenate was filtered through cotton cloth and centrifuged at 45,000 x g for 30 minutes. The supernatant was centrifuged at 100,000 x g for 60 minutes. The precipitate was suspended in 10^{-2} M Tris-HCl (pH 7.3) containing 10^{-2} or 10^{-3} M magnesium acetate (10^{-2} M in the case of hypocotyl ribosomes and 10^{-3} M in that of cotyledon ribosomes), and aggregates were removed by the centrifugation at 13,000 x g for 10 minutes. The supernatant was centrifuged at 100,000 x g for 60 minutes and ribosomes were precipitated. The precipitate was resuspended in the buffer and the aggregates were removed by the centrifugation at 13,000 x g for 10 minutes. This supernatant (ribosome suspension) was used as the crude ribosomes.

Ultracentrifugal Analysis of Ribosomes

The crude ribosomes were again washed with Tris-HCl (pH 7.3) containing 10^{-2} M magnesium acetate and 10^{-2} M KCl, and finally suspended in the same buffer for the ultracentrifugal analysis. A Spinco ultracentrifuge Model E was used. The run was 37,020 rpm at 4°.

Purification of Ribosomes by the Sucrose Density Gradient Centrifugation

The procedures were the same as shown by Britten[22]. On the 30 ml of 5 to 20 % linear sucrose density gradient solution 2 ml of crude ribosome suspension was placed. After the centrifuga-

tion at 24,000 rpm for 3 hours in Spinco SW-25 rotor, the solution was taken out in small portions. The optical density of each fraction was measured at 260 mμ, and the ribosome fraction was collected. This preparation was used as purified ribosomes. An example of the experiments is shown in Fig. 1.

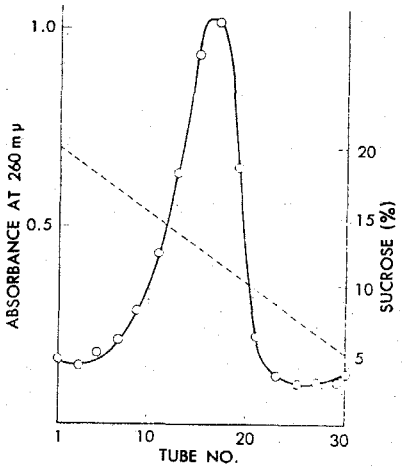

Fig. 1. Fractionation of hypocotyl ribosomes by the sucrose density gradient centrifugation.

Assay Methods

Protein was determined by Lowry method[23]. Ribosomal RNA was determined by the orcinol method[24] after the ribosomal suspension had been dialyzed against 5 x 10^{-3} M Tris-HCl (pH 7.3) containing 10^{-4} M magnesium acetate for 2 days.

Measurement of Enzyme Activity

RNase (EC. 2.7.7.16)— The method was the same as described by Matsushita[25]. 2 ml of the reaction mixture contained 5 mg of RNA, 200 μmoles of sodium acetate (pH 6.0), 20 μmoles of EDTA and the enzyme.

PDase (EC. 3.1.4.4)— The method was the same as described by Matsushita et al.[26]. 2 ml of the reaction mixture contained 2 μmoles of DNPP sodium salt, 200 μmoles of sodium acetate (pH

6.0), 20 μmoles of NaF and the enzyme.

Acid phosphatase (EC. 3.1.3.2)— 2 ml of the reaction mixture contained 5 μmoles of pNPP, 200 μmoles of sodium acetate (pH 5.0), 20 μmoles of magnesium acetate and the enzyme. After incubation for 30 minutes, the reaction was stopped by the addition of 2 ml of 0.4 N sodium hydroxide solution. The mixture was centrifuged to eliminate the cloudiness, and the optical density of the supernatant was read at 420 mμ.

Nucleotidase (EC.3.1.3.5) and nucleoside triphosphatase (EC. 3.6.1.3)— For the determination of nucleotidase, AMP, GMP, CMP, UMP, dGMP and dCMP were used as the substrates. For the determination of nucleoside triphosphatase, ATP, GTP and CTP were used as the substrates. 1 ml of the reaction mixture contained 1 μmole of substrate, 100 μmoles of sodium acetate (pH 5.0), 10 μmoles of magnesium acetate and enzyme. The reaction was stopped by adding 1 ml of 3 N sulfuric acid after 30 minutes. Pi released was measured by Allen's method[27].

Peroxidase (EC.1.11.1.7)— Guaiacol method was used[28]. 3 ml of the reaction mixture contained 5 μmoles of guaiacol, 3 μmoles of hydroperoxide, 300 μmoles of sodium acetate (pH 6.0) and the enzyme. The reaction was performed in a cuvette having 1 cm light path, and the optical density at 470 mμ was read.

β-Glucosidase (EC. 3.2.1.21)— 2 ml of the reaction mixture contained 2 μmoles of pNPG[29], 200 μmoles of sodium acetate (pH 5.0) and the enzyme. After 30 minutes, 2 ml of sodium hydroxide solution was added to stop the reaction and the optical density at 400 mμ was read. The values were calculated from the molar extinction coefficient, 18.14×10^3.

The other enzymes— The following enzyme activities were not found in the ribosomes; the assay methods were as follows. Succinic dehydrogenase (EC. 1.3.99.1) was measured manometrically by using phenazine methosulfate as an electron acceptor[30]. Lactic dehydrogenase (EC. 1.1.1.27) was measured by using NAD as an electron acceptor[31]. Amino acid activating enzyme (EC. 6.1.1) was measured by hydroxamate method[32]. Aldolase (EC. 4.1.2.7) was measured by determining alkali labile phosphate of triose phosphate which was produced by the degradation of FDP[33], and also determined spectrophotometrically by determining DNP hydrazone[34]. Soluble starch and amylopectin were used as the substrate for the determination of amylase (EC. 3.2.1.1, 3.2.1.2)[35]. Leucyl-glycine peptidase was measured by the method of Matheson [36]. Activities of these enzymes were examined at pH 4 to 9.

Enzyme Unit

The unit of the enzyme activity was taken as the amount catalyzing the formation of 1 μmole of the product per minute under the conditions described above. The product of each enzyme reaction was acid soluble nucleotide for RNase, p-nitrophenol for PDase, β-glucosidase and acid phosphatase, Pi for nucleotidase and nucleoside triphosphatase, and diguaiacol for peroxidase. All enzyme reactions were carried out at 37°except that peroxidase was estimated at 20°. Usually, for enzyme assay approximately 60 to 300 μg of ribosomes were used in a tube.

3. RESULTS

Components of Ribosomes from Soybean Cotyledons and Hypocotyls

Fig. 2 shows the results of the ultracentrifugal analysis

of the ribosomes prepared from resting-cotyledons and hypocotyls of 5 day-old seedlings. The main components of the ribosomes from resting-cotyledons and hypocotyls show the same sedimentation velocity. The size was estimated as 78s (S_{20}). A heavy component sedimented prior to the main peak was found in the ribosome fraction from hypocotyls. The size was estimated as 116s. This heavy component was not found in the ribosomes from resting-cotyledons. The heavy components were also found in the

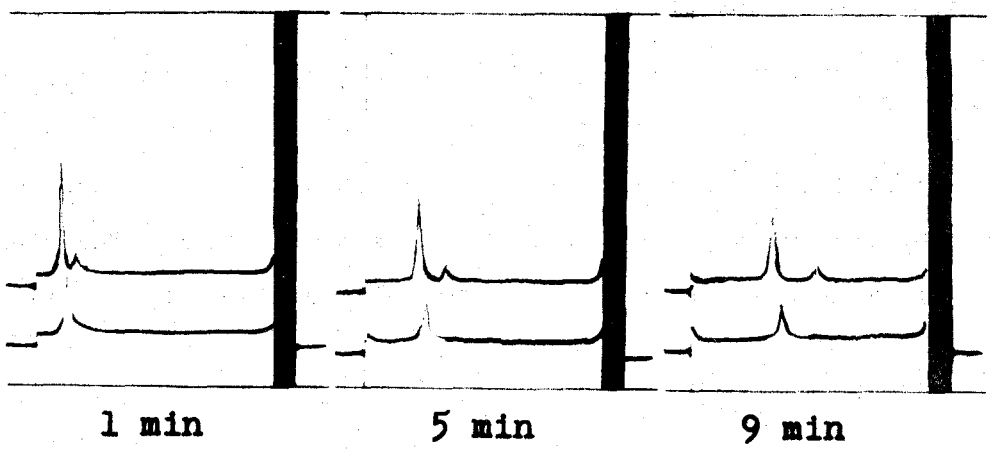

Fig. 2. Patterns of resting-cotyledon ribosomes and hypocotyl ribosomes by the ultracentrifugal analysis.

Ribosomes were suspended in the buffer as shown in the MATERIALS AND METHODS. The photographs show the sedimentation patterns of ribosomes centrifuged for 1, 5 and 9 minutes after the speed reached to 37,020 rpm. Ribosome concentration was 2 %. Upper pattern shows hypocotyl ribosomes and the lower cotyledon ribosomes.

ribosomes from the cotyledons of 5 day-old seedlings as shown in Fig. 3. The main peak was estimated as 78s, and the heavy components were estimated as about 115s and 150s , respectively. The heavy components seemed to be contained in the ribosomes prepared from working tissues such as hypocotyls and cotyledons of 5 day-old seedlings.

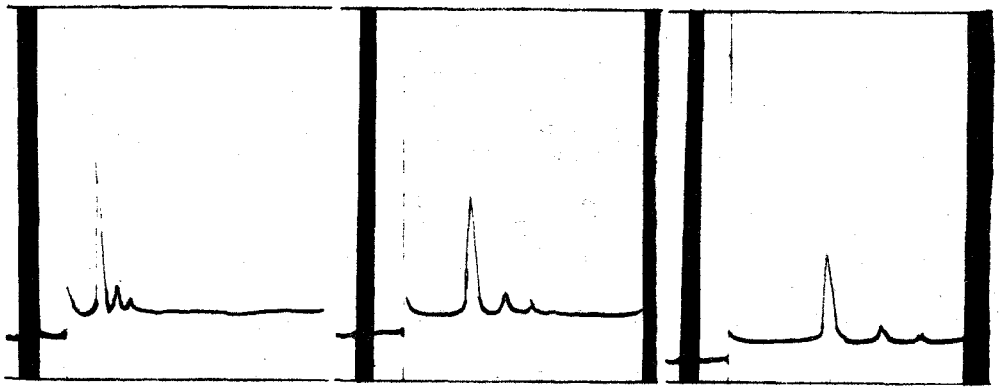

Fig. 3. Patterns of working-cotyledon ribosomes by the ultracentrifugal analysis.

The ribosomes were prepared from the cotyledons of 5 day-old seedlings. The analytical conditions were the same as shown in Fig. 2.

TABLE 1 shows the analysis of the ribosomes which were purified by the sucrose density gradient centrifugation. The ribosomes from hypocotyls contained higher RNA content than resting-

TABLE 1. PROTEIN AND NUCLEIC ACID CONTENT OF PURIFIED RIBOSOMES

Ribosomes	Protein	Ribonucleic acid	OD_{260}/OD_{240}
	%	%	
Cotyledon ribosomes			
Resting	53.7	46.3	1.3 (0.95)
Working	56.3	43.7	〃 (〃)
Hypocotyl ribosomes	44.4	55.6	1.5 (1.0)

Resting-cotyledon ribosomes were prepared from soybeans which were soaked overnight in water. Working-cotyledon ribosomes and hypocotyl ribosomes were prepared from 5 day-old seedlings. These crude ribosomes were purified by the sucrose density gradient centrifugation as shown in MATERIALS AND METHODS. The purified ribosome suspension was dialyzed against buffer as shown in MATERIALS AND METHODS. The values in the parenthesis show the OD ratio of crude ribosomes.

and working-cotyledon ribosomes. The differences were about 10 per cent.

Enzymes Contained in the Purified Ribosomes

TABLE II. ENZYME ACTIVITIES FOUND IN PURIFIED RIBOSOMES

Enzymes	Activities (units × 10^3/mg protein)		
	Resting-cotyledon ribosomes	Working-cotyledon ribosomes	Hypocotyl ribosomes
RNase	3.3	10	109
PDase	4.8	5.0	47
Acid phosphatase	35	93	243
5′-Nucleotidase			
AMP	9.1	44	186
GMP	6.7	42	193
CMP	7.4	38	173
UMP	5.1	36	158
dGMP	7.0	50	145
dCMP	4.7	47	144
XTPase			
ATP	20	35	149
CTP	16	33	126
Peroxidase	43	8.2	67
β-Glucosidase	5.5	2.6	3.3

The ribosomes used were the same as shown in TABLE I except that the preparations were not dialyzed.

The purified ribosomes, prepared by the sucrose density gradient centrifugation, contained several enzyme activities as shown in TABLE II. Hypocotyl ribosomes contained higher enzyme activities than the resting-cotyledon ribosomes. The activities were 20 to 30 times higher with RNase and 5′-nucleotidase and

TABLE III. DISTRIBUTION OF ENZYME ACTIVITIES IN THE SUPERNATANT AND THE RIBOSOMAL FRACTIONS FROM SOYBEAN SEEDLINGS

Enzymes	Activities (units×10^3/mg ribosomal protein)								
	Resting-cotyledon			Working-cotyledon			Hypocotyl		
	S.	R.	S/R	S.	R.	S/R	S.	R.	S/R
RNase	11	1.5	7.3	180	6.4	28	500	102	4.9
PDase	26	3.3	7.9	215	4.4	49	224	136	1.6
Acid phosphatase	496	27	18	7,535	98	77	3,850	325	12
5′-Nucleotidase									
AMP	46	5.1	9.0	764	18	42	343	42	8.2
CMP	34	3.2	11	643	16	40	304	42	7.2
dGMP	44	5.6	7.9	794	18	44	371	49	7.6
dCMP	38	4.9	7.8	742	18	41	359	52	6.9
XTPase									
ATP	149	16	9.3	3,920	49	80	3,465	175	20
CTP	113	13	8.7	2,860	50	57	2,680	201	13
Peroxidase	79	24	3.3	417	14	30	1,778	298	6.0
β-Glucosidase	6.1	1.8	3.4	14	0.8	16	22	2.0	11

Enzyme activities of the supernatant (cytoplasmic solution) were calculated as the units per mg of the ribosomal protein obtained from the homogenate. Therefore, S/R shows the comparison of the distribution of enzymes in the supernatant (cytoplasmic solution) and ribosomes. Recovery of the ribosomes was about 1.1 mg per g wet weight of both resting- and working-cotyledons and 0.32 mg per g wet weight of hypocotyls. Resting-cotyledons were prepared from soybeans soaked overnight in water, and working-cotyledons and hypocotyls were separated from 5 day-old seedlings. S., supernatant; R., ribosomes.

about 7 times with acid phosphatase and XTPase. Only β-glucosidase activity of hypocotyl ribosomes was lower than that of cotyledon ribosomes.

Working-cotyledon ribosomes prepared from 5 day-old seedlings

contained higher enzyme activities than resting-cotyledon ribosomes, but lower than the hypocotyl ribosomes. The activities in working-cotyledon ribosomes were about 3 times higher with RNase and acid phosphatase, 5 to 10 times with 5′-nucleotidase and 2 times with XTPase than resting-cotyledon ribosomes. However, peroxidase and β-glucosidase were lower. The experiments could be repeated with similar results.

Succinic dehydrogenase, lactic dehydrogenase, amino acid activating enzyme, aldolase, amylase and leucyl-glycine peptidase which had been reported to be found in some other ribosomes were measured at pH 4 to 9, but any activities were not found in the ribosomes from soybeans.

The distribution of the enzymes in the crude ribosomes and the supernatant (cytoplasmic solution) is shown in TABLE III. The enzyme activities were higher in the cytoplasmic solution than in ribosome fraction. In the resting-cotyledons, peroxidase and β-glucosidase were comparatively rich in ribosome fraction. About 30 % of the total peroxidase or β-glucosidase activity in the cell was found in ribosome fraction, on which the other enzymes were located about 10 %. In the hypocotyls, about 20 % of RNase and 40 % of PDase were found in the ribosome fraction.

Comparing TABLEs II and III, it is seen that specific activities of resting-cotyledon ribosomes were increased by the purification, while in the case of hypocotyl ribosomes, specific activity of 5′-nucleotidase was increased and those of PDase, acid phosphatase and peroxidase decreased.

Changes in the Enzyme Activities during Germination

Cotyledons and hypocotyls were separated from seedlings, and each part was homogenized and centrifuged. The enzyme activities of the respective supernatant were measured. Fig. 4 shows the changes in the enzyme activities of hypocotyls of 2 day to 6 day seedlings. The values were shown as the relative values to the activities of 2 day seedlings. In the hypocotyls, the increases of the enzyme activities were not large compared with those of cotyledons. The enzyme activities per cell of the hypocotyls

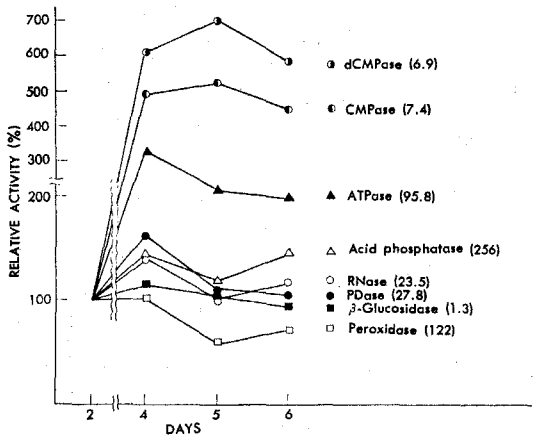

Fig. 4. **Changes of enzyme activities** in the hypocotyls during germination of soybean.

The hypocotyls were homogenized with sucrose solution and centrifuged at 35,000 x g for 30 minutes. The supernatant was used for the enzyme assay. The enzyme activities were compared as units per g protein of the hypocotyls. The activities of the hypocotyls from 2 day-old seedlings were regarded as 100. The values in the parenthesis show the units of the enzyme activities of the hypocotyls from 2 day-old seedlings (units/g protein).

may be almost constant in the course of germination.

Fig. 5 shows the enzyme activities in the cotyledons during germination. With the progress of germination, activities of RNase, PDase and peroxidase increased 3 to 5 times, but absolute values per g protein of the tissue were lower than those of hyp-

ocotyls. In contrast, ATPase, nucleotidase such as CMPase or dCMPase and acid phosphatase increased 10 to 25 times. The absolute values of those enzymes were higher than those of hypocotyls.

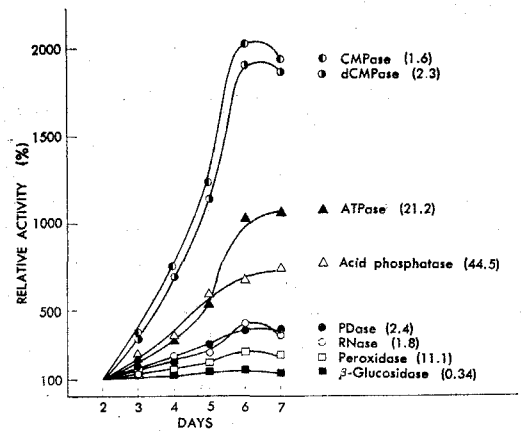

Fig. 5. Changes of enzyme activities in cotyledons during germination of soybean.

Cotyledons were detached from seedlings. The cotyledons were homogenized with sucrose solution and centrifuged at 35,000 x g for 30 minutes. The enzyme activities were obtained as units per g protein. The activities are shown as the percentage to those of the cotyledons from 2 day-old seedlings. The values in the parenthesis show the activities (units/g protein) of the cotyledons from 2 day-old seedlings.

Test of Adsorption of the Enzyme Contained in the Cytoplasmic Solution onto the Resting-Cotyledon Ribosomes

The supernatant, the cytoplasmic solution prepared from cotyledons and hypocotyls of 5 day-old seedlings, contained high enzyme activities as shown in TABLE III, Fig. 4 and Fig. 5. With this supernatant, resting-cotyledon from soybeans soaked overnight in water were homogenized, and ribosomes were prepared. The ribosomes prepared by treating with the supernatant from cotyledons of 5 day-old seedlings contained about twice higher activities of RNase, acid phosphatase, AMPase, and ATPase comp-

aring with the original resting-cotyledon ribosomes, and the activities reached the level close to that of working-cotyledon ribosomes. On the other hand, the ribosomes prepared by treating with the supernatant from hypocotyls did not show any increase of the enzyme activities, that is, no adsorption of the enzymes were observed (TABLE IV).

TABLE IV. ADSORPTION OF ENZYMES ONTO THE SURFACE OF RESTING-COTYLEDON RIBOSOMES

Enzymes	Activities (units x 10^3/mg ribosomal protein)		
	Orig. R.[a]	Treated with S. of cotyledons[b]	Treated with S. of hypocotyls[c]
RNase	1.7	3.7	2.2
PDase	3.0	4.4	4.0
Acid phosphatase	31	64	37
AMPase	7.0	20	12
ATPase	17	32	20

a Original resting-cotyledon ribosomes prepared from the cotyledons of soybeans soaked overnight in water by homogenizing with the sucrose solution as shown in MATERIALS AND METHODS.
b Prepared by homogenizing the resting-cotyledons (60 g) with the supernatant obtained from the homogenate of cotyledons of 5 day-old seedlings (60 g).
c Prepared by homogenizing the resting-cotyledons (60 g) with the supernatant obtained from the homogenate of hypocotyls of 5 day-old seedlings (60 g).

4. DISCUSSION

In Figs. 4 and 5, enzyme activities are shown per g protein of the tissues. The increase of the enzyme activities in the hypocotyls was not large when compared with that of cotyledons. The enzyme activities per g protein of the hypocotyls may be almost constant after 4 days of germination (Fig. 4). Enzyme activities increased more in the cotyledons with germination (

Fig. 5). The increase of some enzyme activities in cotyledons or endosperms has also been reported with pea seedlings[15] or barley endosperms[16]. Not only hypocotyls but also cotyledons of seedlings can be regarded as working tissues. The activities of the ribosome systems increased to a maximum in peanut cotyledons of the imbibed seed from a low level in the dry seed[12].

When resting-cotyledon ribosomes and working-ribosomes from the cotyledons and hypocotyls of 5 day-old seedlings were compared, the resting-cotyledon ribosomes were found to consist of only 80s particles but the working-ribosomes contained larger 115s and 150s particles, as shown in Fig. 2 and Fig. 3. These particles may correspond to the polymers of ribosomes or polysomes which have been found in reticulocytes[37, 38], pea seedlings[39] and cabbage leaves[40].

The main fraction, 80s, of the resting-cotyledon, working-cotyledon and hypocotyl ribosomes was prepared by the sucrose density gradient centrifugation. Hypocotyl ribosomes showed higher RNA content than both resting- and working-cotyledon ribosomes. The enzyme activities were also high in the hypocotyl ribosomes. The ratio of OD_{260}/OD_{240} of ribosomes is considered as a criterion of the purity. Those of hypocotyl ribosomes and cotyledon ribosomes were 1.5 and 1.3, respectively (TABLE I) while that of rabbit liver ribosomes was 1.6[41]. The value of OD_{260}/OD_{240} of ribosomes reached the maximum (those of ribosomes purified by the sucrose density gradient centrifugation) by washing the crude ribosomes twice with the buffer containing magnesium ions. The concentration of magnesium acetate used for washing was 10^{-2} M for hypocotyl ribosomes and 10^{-3} M for cotyledon

ribosomes. These concentrations were determined to be optimun for ribosome recovery. Hypocotyl ribosomes contained 20 to 30 times higher activities than resting-cotyledon ribosomes with RNase and 5′-nucleotidase, and 10 times with PDase, acid phosphatase and XTPase. Working-cotyledon ribosomes showed higher activities than resting-cotyledon ribosomes.

In the hypocotyls, the enzyme activities were high (TABLE III) in the cytoplasmic solution (supernatant), and high enzyme activities were also found in the ribosomes. This fact may be interpreted as caused by (a) newly synthesized enzymes which were not released from the ribosomes yet, or (b) soluble enzymes adsorbed on the ribosome surface which were solubilized on disruption of the cell. However, in contrast to these results, acid phosphatase, 5′-nucleotidase and XTPase activities were higher in the supernatant of working-cotyledons than in that of hypocotyls (TABLE III, S/R), although the activities were low in the ribosomes. And also the ribosomes prepared from resting-cotyledons by treating with the supernatants of hypocotyls did not show increased enzyme activities suggesting that the adsorption of the enzymes on the ribosomes is unlikely in this case. That is, the enzymes in the supernatant of working-cotyledons were adsorbed to the resting-cotyledon ribosomes but the enzymes in the supernatant of hypocotyls were not (TABLE IV). The specific activities of some enzymes were increased by washing, and some enzymes were concentrated in ribosomes.

It has been reported in E. coli[42] and pea seedlings[43] that XTPase activities were higher in polysome fractions which were active for amino acid incorporation. On the other hand,

there have been several reports on enzymes at the bacterial cell surface[44-46]. Though the RNase foune in the ribosomes from E. coli was believed to be a constituent of the ribosomes[19], it was recently reported that the enzyme occurs near or at the cell surface and may be adsorbed on the ribosomes after being solubilized on disruption of the cell[44]. These facts may show that the ribosomal enzyme activities can not be attributed to only one of the three status of the ribosomal enzymes described in the INTRODUCTION.

CHAPTER III

AMINO ACID INCORPORATING SYSTEMS FROM SOYBEAN SEEDS AND SEEDLINGS[b)]

1. INTRODUCTION

Protein synthesis has been demonstrated with ribosomal systems of animal and bacterial origins[1-3)]. Similar systems from higher plants appeared to be active[4-14)]. The concept that polysomes are the primary sites of protein synthesis[47)] has also been accepted from some findings in plants; new RNA synthesis in roots excised from pea seedlings[48)], finding of polysomes in leaves[49)] and activation of protein synthesizing systems in imbibed peanut cotyledons[11, 12 50)]. It has been shown that the activities of the ribosomal amino acid incorporating systems increased from a low level in the dry seeds to a maximum in the imbibed seeds. This increase in activity is associated with polysome formations[12)].

In this chapter, protein synthesizing activities of the ribosome preparations from the cotyledons of soybeans soaked overnight in water (resting-cotyledon ribosomes) and from the cotyledons and the hypocotyls of the growing seedlings (working-cotyledon and hypocotyl ribosomes) are compared. And it is shown that the amino acid incorporating activities of ribosome preparations may depend on the amount of polysomes contained.

2. MATERIALS AND METHODS

Preparation of Ribosomes

Soybean seeds were germinated in vermiculite at 25°in the

dark. Hypocotyls and cotyledons were separated from the seedlings, and ribosomes were prepared from the respective organs. Zero-coty (resting-cotyledon) ribosomes were prepared from cotyledons separated from soybeans soaked overnight in water at 4°. The materials were ground in a mortar with 0.05 M Tris-HCl (pH 7.8) containing 0.25 M sucrose, 0.001 M magnesium acetate and 0.005 M 2-mercaptoethanol (4 ml/g tissues). The homogenate was squeezed through gauze, and the filtrate was centrifuged for 30 minutes at 10,000 x g. The supernatant was centrifuged at 100,000 x g for 60 minutes. The precipitate was suspended in 0.01 M Tris-HCl (pH 7.8) containing 0.001 M magnesium acetate and 0.005 M 2-mercaptoethanol. The supernatant (ribosome suspension) was used as the crude (once pelleted) ribosome preparation. All procedures were carried out in a cold room.

Fractionation fo Ribosomes by Sucrose Density Gradient Centrifugation

The crude ribosomes were centrifuged in a Hitachi RPS 25A swing bucket rotor for 180 minutes at 24,000 rpm on a 15-35 % linear sucrose density gradient, containing 0.05 M Tris-HCl (pH 7.8), 0.001 M magnesium acetate and 0.005 M 2-mercaptoethanol. Each fractions from 25 to 30 drops were collected in 30 tubes and analyzed after appropriate dilution with water.

Preparation of sRNA

Three or 4 day-old seedlings (whole tissues) were ground in a mortar with the same buffer solution as used for preparing ribosomes. The homogenate was centrifuged for 60 minutes at 100,000 x g. From this supernatant, RNA was prepared by the phenol method[51]. The RNA preparation was dialyzed against

water and used for the incorporating system.

Preparation of Supernatant

The 100,000 x g supernatant mentioned above (4 ml) was charged on a Sephadex G-25 column (20 ml), and eluted with 0.05 M Tris-HCl (pH 7.8) containing 0.005 M 2-mercaptoethanol. The protein fraction which passed through first was used as the "supernatant".

Measurement of Amino Acid Incorporation

The complete incubation system for determining amino acid incorporation was consisted of 50 μmoles Tris-HCl (pH 7.8), 50 μmoles potassium chloride, 5 μmoles magnesium acetate, 1 μmole ATP, 10 μmoles creatine phosphate, 50 μg creatine kinase, 0.25 μmole GTP, 10 μmoles 2-mercaptoethanol, 240 μg sRNA (from 3 or 4 day-old seedlings unless otherwise stated), 20 mμmoles each of 19 amino acids, 4.7 mμmoles leucine-^{14}C and designated amount of ribosomes. In the standard assay system, the "supernatant" (from 3-hypo unless otherwise stated) was supplemented to the complete incubation mixture. Unless otherwise indicated, the reaction mixture (a total volume of 1 ml) was incubated in a test tube at 37°. After 20 minutes, the reaction was stopped by adding 1 ml of cold 10 % TCA solution. The precipitate was washed twice with cold 5 % TCA and boiled for 15 minutes with 5 % TCA, and again washed with cold 5 % TCA. The washed precipitate was dissolved in 1 N ammonia and dried in a planchet. The radioactivity was counted with a gas flow counter.

Protein Determination

Protein was determined by the method of Lowry et al.[52].

Preparation of ^{32}P-labeled Ribosomes

Whole 4 day-old seedlings (13 g) and isolated 0-and 4-coty

(30 g each) were soaked in a 30 ml solution containing 10 μg ^{32}P-orthophosphate (pH was adjusted at 6.0 with KOH), respectively. Hypocotyl and cotyledon ribosomes were prepared as described above and ^{32}P incorporated was measured with a GM counter.

Reagents

ATP and creatine phosphate were obtained from Sigma Chemical Co., GTP from Pabst Laboratories, creatine kinase from Boehringer and Soehne Co., leucine-^{14}C (uniformly labeled, 214 μc/μmole) from Daiichi Chemical Co., and ^{32}P-orthophosphate (99 c/mg) from Radioactive Center in England.

3. RESULTS

Requirement for the Amino Acid Incorporation

TABLE V shows the characteristics of the amino acid incorporating system. The requirement for reaction components was similar to that of protein synthesizing systems from bacteria and liver[1, 2]. The addition of ATP, GTP, magnesium ions and potassium ions was essential. The lack of requirement for a mixture of amino acids is not unusual in such crude ribosomal systems as used here and has generally been ascribed to the presence of these components as contaminants in the ribosomal fractions[11]. In fact, the addition of crude supernatant apparently inhibited the incorporation of amino acids probably because of the dilution of labeled amino acids by endogenous amino acids in the supernatant. However, when the supernatant pretreated with Sephadex ("supernatant") was added, an increase in the incorporating activity was observed. Therefore, the "supernatant" was included in the standard assay system. The ribosomes should be

TABLE V. REQUIREMENT FOR LEUCINE INCORPORATION INTO SOYBEAN HYPOCOTYL RIBOSOMES

System	Incorporation cpm/mg protein
Complete	2380
minus ATP and ATP generating system[a]	58
minus GTP	203
minus sRNA	1072
minus amino acid mixture	2290
minus KCl	1014
minus magnesium acetate	87
plus supernatant (crude)	696
plus "supernatant" (Sephadex treated)[b]	4120
plus "supernatant" and minus ribosomes	0

The complete system is described under MATERIALS AND METHODS. The specific activity is shown as total leucine-^{14}C incorporated in 20 minutes per mg protein. The amounts of ribosomes and the supernatant were 0.35 mg and 1.43 mg protein per tube, respectively.
a; creatine phosphate and creatine kinase.
b; standard assay system.

TABLE VI. EFFECTS OF ANTIBIOTICS ON AMINO ACID INCORPORATING SYSTEM

Addition	Relative activity (%)
Control	100
Chloramphenicol	87
Puromycin	23
Streptomycin	95

The standard assay system was used. The amount of ribosomes was 0.47 mg protein and that of the supernatant was 1.57 mg protein per tube. The amount of antibiotics was 50 µg per tube.

prepared by grinding tissues in a mortar by hand. When a blender was used, the incorporating activity was lost. The amino acid incorporating system was inhibited greatly with puromycin but to a lesser extent with chloramphenicol or streptomycin (TABLE VI).

Activities of the Ribosomes and the Supernatant from Different Parts of Soybean Seedlings

Activities of the ribosomes and the supernatant from different parts of seedlings were compared (TABLE VII). Ribosomes from 3-coty, 5-coty and 3-hypo showed some activities in incorporating amino acids even though no "supernatant" was added. The "supernatant" from 0-coty, 3-coty, 5-coty or 3-hypo greatly enhanced the incorporation of amino acids into the ribosomes from 3-hypo. The "supernatant" from 3-coty was most effective, and that from 0-coty next. Almost any activity was not detected for the 0-coty ribosomes irrespective of the addition of the most active "supernatant". The activity of ribosomes from 3-coty and 5-coty was increased by the "supernatant" added.

TABLE VII. COMPARISON OF THE RIBOSOMES AND THE SUPERNATANT FROM DIFFERENT PARTS OF SOYBEAN SEEDLINGS

Ribosomes	Amino acid incorporated (cpm/mg ribosomal protein)				
	"Supernatant"[a]				
	none	3-hypo	0-coty	3-coty	5-coty
3-hypo	1160	2030	6150	8180	2900
0-coty	29			58	
3-coty	696			2000	
5-coty	203			812	

The standard assay system was used. The amount of ribosomes used as protein content was 0.34 mg for 3-hypo and about 1.5 mg for 0-coty, and the amount of the "supernatant" as protein was 1.3 mg for 3-hypo and 3.2 mg for cotyledons, respectively.
a, supernatant treated with Sephadex

Effects of sRNA from Different Tissues

As shown in TABLE VIII, sRNA from 0-coty was most active, and that from 4-hypo was rather inactive. In the amino acid

TABLE VIII. EFFECT OF sRNA PREPARATIONS PREPARED FROM 0-COTY, 4-COTY AND 4-HYPO

Assay system	Source of sRNA	^{14}C-leucine incorporated (cpm/mg ribosomal protein)
Standard assay system[a] less supernatant	less sRNA	696
〃	0-coty	2490
〃	4-coty	2090
〃	4-hypo	580
Standard assay system[a]	less sRNA	4265
〃	0-coty	10040
〃	4-coty	9370
〃	4-hypo	4295

The amount of ribosomes and the supernatant expressed as protein contained, and sRNA were 0.6 and 2.4 mg, and 240 μg per tube, respectively.
a, Standard assay system described in MATERIALS AND METHODS.

incorporating system from maize[9], sRNA was reported to inhibit leucine incorporation, and this inhibitory action was eliminated by dialyzing sRNA against 10^{-4} M EDTA and 10^{-3} M cacodylate buffer at pH 7.0[9]. The similar phenomenon was also reported on mammalian sRNA[53, 54]. However, in the case of sRNA from 4-hypo, no enhancing effect of the dialysis against EDTA was observed.

Sucrose Gradient Sedimentation Profiles of Ribosomes and the Distribution of Amino Acid Incorporating Activities

Ribosomes from 3-hypo and 3-coty were subjected to sucrose gradient sedimentation. A portion of each 25-30 drops fractionated was removed to be assayed for the optical density at 260 mμ. The residual aliquots were pooled as fractions I to V as shown in Fig. 6. The amino acid incorporating activities of these fractions were determined. The peak of OD curve at 260 mμ shown in Fig. 6 corresponds to 80s particles as described in CHAPTER II. The fraction I-IV which are heavier than monosomes

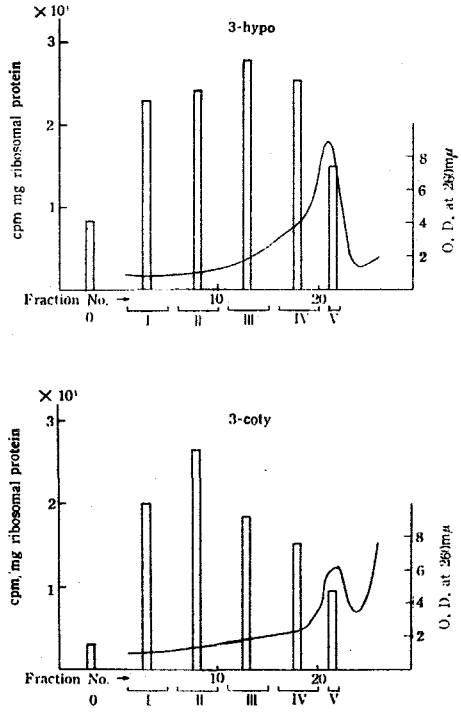

Fig. 6. Sucrose gradient sedimentation profiles of ribosomes prepared from 3-hypo and 3-coty, and their amino acid incorporating activities.

The curve shows OD at 260 mμ. The vertical columns show the specific radioactivities per mg ribosomal protein. Fraction I- V were the pooled fractions. Fraction 0 is the original (before density gradient centrifugation) ribosomes. The standard assay system was used for amino acid incorporation.

correspond to polysome regions as having been shown for reticulocyte polysomes[55]. Polysome fractions showed higher specific activities of amino acid incorporation than monosome fraction (fraction V). The original (crude) ribosome fraction from cotyledons showed lower activities than those of hypocotyls. However, the polysome fraction from either ribosomal preparation gave very similar specific activity. The difference observed in the activities of the crude ribosomes may be due to the amount of polysomes contained.

Incorporation of ^{32}P-orthophosphate in Ribosome Fractions

Fig. 7 shows the sucrose gradient sedimentation profiles of the ribosomes prepared from ^{32}P-labeled 4-hypo, 0-coty and 4-coty. Incorporation of ^{32}P-orthophosphate in the ribosomes from

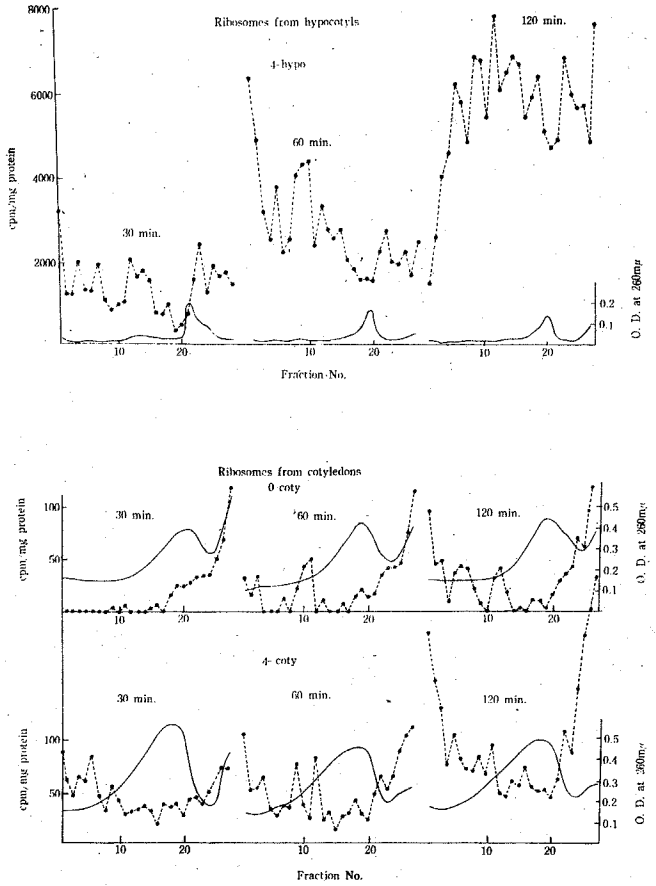

Fig. 7. Sucrose gradient centrifugation profiles of the ribosomes in which ^{32}P-orthophosphate was incorporated.

Full line shows the OD at 260 mμ. Dotted line shows the specific radioactivity per mg protein. The procedures are shown in MATERIALS AND METHODS. Time shows the incubation time.

4-hypo increased as the soaking time was prolonged. The specific radioactivities of phosphorus were high in the polysome

region at the early stages. Although incorporation of ^{32}P in the ribosomes from cotyledons was very low compared with that of hypocotyls after long soaking, a similar tendency of predominant incorporation in the polysome region was observed. The incorporation of ^{32}P in ribosomes is considered to show the synthesis of new RNA. And the predominant incorporation in the polysome region at the earlier stages may suggest that the mewly synthesized RNA was mRNA which have been considered to be necessary for the formation of polysomes. However, the incorporation of ^{32}P in the ribosomes from 0-coty was very low compared with those of 4-coty (working). RNA synthesizing system may be dormant and polysomes may not be contained in the resting cotyledons.

4. DISCUSSION

The present amino acid incorporating system from soybean seedlings appeared to be analogous to the system of animal and bacterial origins[1-3] and to those from other plant tissues as for both the requirements for reaction components and the inhibition with antibiotics[4-14]. Amino acids were incorporated in the ribosomes without supernatant added (TABLE V), and it was considered that the ribosome preparation used contained enzymes necessary for incorporating amino acids although the amount might not be sufficient. The amino acid incorporation was stimulated by adding the "supernatant" treated with Sephadex. The "supernatant" from cotyledons, 0- to 5-coty, was effective. On the other hand, ribosomes from 0-coty was inactive in incorporating amino acids even though a sufficient amount of "supernatant" was added. This fact suggests that polysomes were not contained

in ribosome fraction from 0-coty. It seems that 80s ribosomes in 0-coty itself may be deficient in the ability to form polysomes or mRNA may not be contained in 0-coty. However, sRNA from 0-coty was active as shown in TABLE VIII. Ribosomes from 3- and 5-coty were somewhat active although the activities were lower than that of hypocotyls. This fact was coincident with the finding of Marcus and Feeley[12].

As shown in Fig. 6, the polysome regions separated by sucrose gradient centrifugation showed a higher amino acid incorporating activities than the monosome fraction. The crude ribosome fraction from 3-coty was lower in activity than that from 3-hypo, however, polysome fractions from both 3-coty and 3-hypo showed a similar specific activity. From these results, it was deduced that the incorporating activity may depend on the content of polysomes in the crude ribosome fractions. Fig. 2 shows that labeled phosphorus was incorporated in the polysome region at the early stages when cotyledons and seedlings were immeresed into ^{32}P-orthophosphate solution. The predominant incorporation in the polysome region may suggest the new synthesis of mRNA[47-50]. Incorporation of phosphorus into RNA of 0-coty was rather low compared with that of active tissues. RNA synthesizing system may be still dormant in the resting cotyledons. However, the formation or activation of mRNA occurs during seed germination.

In these experiments, the possibility of bacterial contamination was considered to be negligible. Because, (1) bacterial bodies can be precipitated at the centrifugation to eliminate mitochondria, (2) shape of the time course for incorporating

activity always slope off after 60 minutes which would not be expected for active bacteria, and (3) ATP and its generating system was necessary for the reaction.

At any rate, the present study indicated that the activities of the ribosome systems increase as the seeds imbibe, that is, germination of seeds likely parallels with the activation of the protein synthesizing systems in cotyledons.

CHAPTER IV

OCCURRENCE OF THE RIBONUCLEIC ACIDS THAT HAVE TEMPLATE ACTIVITIES IN SOYBEAN SEEDS[c)]

Physiological changes occurring in seeds during germination, the changes of several enzyme activities in the ribosomes from soybean seedlings (CHAPTER II) and the activation of amino acid incorporating system during germination (CHAPTER III), was described. During the course of preparing tRNAs from soybean cotyledons, RNAs having template activities were found in the 100,000 x g supernatant fraction (S-100), which was free from ribosomes and polysomes.

Soybean seeds soaked overnight in water were used. The tissues were ground in a mortar with 0.05 M Tris-HCl (pH 7.8) containing 0.25 M sucrose, 0.001 M magnesium acetate, and 0.005 M 2-mercaptoethanol. After centrifugation of the homogenate at 10,000 x g for 30 minutes, further centrifugation at 100,000 x g for 2 hours was carried out to obtain the supernatant fraction (S-100). Ribonucleic acid was prepared from S-100 by phenol method[51)] (S-100 RNA).

The S-100 RNAs were active in stimulating an amino acid incorporating system obtained from soybean seedlings prepared by the method described in CHAPTER III. As shown in Fig. 8, S-100 RNAs were fractionated into high (Fraction I) and low (Fraction II) molecular weight RNAs with a Sephadex G-200 column. Fractions I and II showed stimulating effects on the amino acid incorporating system, respectively (TABLE IX). Both RNA preparations were prepared from the 100,000 x g supernatant fraction, but the

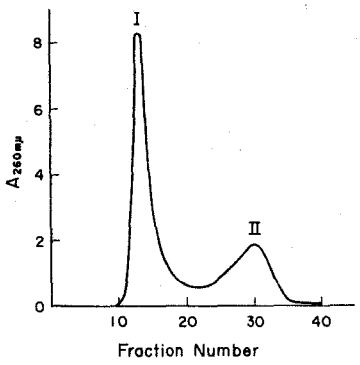

Fig. 8. Separation of S-100 RNAs on a Sephadex G-200 column.

The solution of S-100 was applied to the Sephadex G-200 column (1.4 x 50 cm) and eluted with 2 % K acetate adjusted pH 6. Each 2 ml fraction was collected and the absorbance at 260 mμ was measured. Fractions 11-17 and 25-33 were collected as Fraction I (high molecular RNA) and II (low molecular RNA), respectively.

TAB E IX. EFFECTS OF RIBONUCLEIC ACIDS ON THE AMINO ACID INCORPORATING SYSTEM FROM SOYBEAN AND SOYBEAN SEEDLINGS

RNA's	Leucine-^{14}C-incorporated (cpm/mg ribosomal protein)			
	I	II	III	IV
None	4185	2010	2580	2050
S-100 RNA	13,560	9270	7340	
Fraction I	12,750	5575		
Fraction II	11,770	3890		
Hypocotyl rRNA			3570	3935
Cotyledon rRNA			5250	3935

The assay system is the same as described in CHAPTER III. The preparative methods for S-100 RNA and Fractions I and II are described above and Fig. 8. Ribosomal RNAs were prepared from 4 day-old seedlings by the phenol method; 250 μg of each RNA was added. After incubation for 20 minutes at 37°, the hot acid-insoluble material was counted by the method described in CHAPTER III.

nature of these RNAs differed distinctly in their molecular wei-

ghts and amino acid acceptor abilities. Fig. 9 shows that Fraction I had no amino acid acceptor abilities, while Fraction II indicated remarkable acceptor abilities. The specific activity of this preparation was rather high compared with that of yeast tRNA, which was used as the control (Fig. 9). From these results,

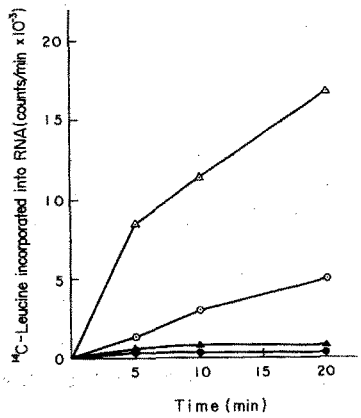

Fig. 9. Amino acid acceptor ability of RNA fractions.

The incubation mixture contained, in 1 ml: 50 μmoles Tris-HCl (pH 7.8), 5 μmoles Mg-acetate, 50 μmoles KCl, 5 μmoles ATP, 1.9 mμmoles leucine-^{14}C (0.4 μc), the supernatant fraction (1 mg protein), and RNA. Yeast tRNA was prepared by the method of Monier et al.[56]; 200 μg of each RNA was added. After incubation at 37°for theindicated times, RNA was precipitated with an ethanol-salt mixture (Berg et al.[57]), washed, and counted. ▲, No addition of RNA; ●, Fraction I; △, Fraction II; ○, yeast tRNA.

it is considered that the stimulating effect of Fraction I for amino acid incorporation may be due to its template activity, and that of Fraction II may depend on the function as tRNA. The yields of Fraction I and Fraction II from cotyledons were about 100 and 25 mg/100 g wet tissues.

Ribosomal RNA also showed stimulating effects to some extent (TABLE IX). This phenomenon, however, may be explained by the presence of mRNA in ribosome or polysome fractions.

The evidence for the template nature of Fraction I was strengthened by using the amino acid incorporating system of E. coli (TABLE X).

TABLE X. EFFECTS OF RIBONUCLEIC ACIDS FROM SOYBEAN COTYLEDONS ON THE AMINO ACID INCORPORATING SYSTEM OF E. COLI

RNA's	Leucine-^{14}C-incorporated (cpm/mg ribosomal protein)
None	1180
S-100 RNA	8990
Fraction I	17,110
Fraction I[a]	12,300
Fraction II	1290

[a] 250 μg RNA was added.

E. coli K-12 was used. The amino acid incorporating system was prepared by the method described by Nirenberg[58]. The S-30 preparation (30,000 x g supernatant fraction) was preincubated for 80 minutes at 37°. The incubation mixture for determining amino acid incorporation was the same as shown in TABLE IX except that the S-30 preparation from E. coli was used as the ribosome and enzyme system, the magnesium concentration was changed to 0.01 M, and the incubation time was 45 minutes. The amount of RNA added was 500 μg.

In this system, high molecular RNA showed very high stimulating effects for the amino acid incorporation into ribosomes.

Since its discovery by Volkin and Astrachan[59], many workers have reported on mRNA prepared from whole cells or polysome fractions of various sources[60]. There is little possibility that these RNAs came from polysomes artifactually during the preparing process. The facts that the yield of the high molecular

RNAs was comparatively high and that the RNAs were obtained from the so-called supernatant fraction are of interest.

The roles of this template-like RNA are still not certain; they may function during the ripening process in the synthesis of reserve proteins or are stores for germination.

CHAPTER V

CHARACTERIZATION OF THE RIBONUCLEIC ACIDS CONTAINED IN THE SOLUBLE FRACTION FROM SOYBEAN SEEDS[d)]

1. INTRODUCTION

Physiological changes in soybean seeds during germination have been shown in the previous chapters. In CHAPTER IV, it was shown that S-100 RNA contained some RNAs having template activities. Those RNAs differed from usual tRNA in the molecular size and amino acid accepting ability.

Template activities and the characteristics of various RNA preparations from mammalian sources have been reported[61-65)]. Recently, the RNA preparation from peanut cotyledons was reported to have a template activity. The fraction which was eluted after ribosomal RNAs on the methylated albumin-kieselguhr column revealed the highest activity[66)].

This chapter deals with further characteristics of RNA that have template activity from soybean seeds. The RNA preparation consisted of two components, and both components stimulated the incorporation of amino acids in a cell-free system of E. coli.

2. MATERIALS AND METHODS

Isolation of RNA

Tissues (cotyledons or hypocotyls detached from soybean seeds or soybean seedlings) were homogenized in a mortar with 0.05 M Tris-HCl (pH 7.8) containing 0.25 M sucrose, 0.001 M magnesium acetate. The homogenate was squeezed through gauze and centrifuged for 30 minutes at 10,000 x g. This supernatant

was centrifuged for 2 hours at 100,000 x g. The supernatant (S-100) was decanted and submitted to the preparation of RNA. The precipitate was suspended with Tris-HCl (0.01 M, pH 7.8) containing 0.001 M magnesium acetate (TM buffer) and centrifuged for 15 minutes at 10,000 x g. This supernatant was centrifuged for 2 hours at 100,000 x g. The precipitate was suspended with TM buffer and used as ribosome suspension. S-100 was treated with aqueous phenol[51]. The RNA was precipitated from the aqueous phase with two volumes of ethanol in the presence of 2 % potassium acetate (pH 5.0). The precipitate was dissolved in water, dialyzed against water and centrifuged for 15 minutes at 10,000 x g. Furthermore, purified RNA was obtained by repeating the same procedure (S-100 RNA). Ribosomal RNA (rRNA) was prepared from the ribosome suspension as the same procedures as S-100 RNA.

S-100 RNA was fractionated into high (H-RNA) and low (tRNA) molecular weight RNAs with a Sephadex G-200 column as shown in CHAPTER IV. All operations were carried out below 4°. H-RNA used in the experiment was that from resting cotyledons if not otherwise stated.

Yeast tRNA was prepared as described by Monier et al.[56] and fractionated by passing through Sephadex G-200. The tRNAs were treated with a mild alkaline solution (0.2 M carbonate buffer, pH 10) at 37°for 1 hour to release esterified amino acids. All RNA preparations were stored at -20°.

Preparation of Preincubated S-30 Fraction of E. coli

E. coli strain K-12 was grown at 20°in a medium containing 10 g of beef extract, 10g of peptone and 5 g of sodium chloride per liter. When an absorbance reached to 0.6 at 520 mμ, the

medium was chilled and the bacteria were harvested by centrifugation, washed and frozen. The cells were ground with alumina and the cell-free extract was prepared. This extract was incubated at 37°for 80 minutes, then dialyzed, divided into portions and stored at -20°as described by Nirenberg[58].

Measurement of Amino Acid Incorporation

The incubation mixture contained 50 μmoles Tris-HCl (pH 7.8), 50 μmoles potassium chloride, 10 μmoles magnesium acetate, 1 μmole ATP, 10 μmoles creatine phosphate, 50 μg creatine kinase, 0.25 μmole GTP, 10 μmoles 2-mercaptoethanol, 20 mμmoles each of 19 amino acids, 4.7 mμmoles leucine-U-^{14}C (1 μc, 214 μc/μmole), preincubated S-30 fraction (0.961 mg as protein) and RNA preparation in a total volume of 1 ml. After the incubation at 37° for 45 minutes, the reaction was stopped by the addition of an equal volume of 10 % TCA. The precipitate was washed and radioactivity was counted as descibed in CHAPTER III. The radioactivity incorporated was proportional to the amount of S-30 fraction, and the data were, therefore, expressed as cpm per mg of protein of S-30 fraction.

Physical Measurements

Sedimentation coefficients were measured in a Spinco Model E ultracentrifuge. The concentration of the H-RNAs used for schlieren analysis were 4.5, 6.5 and 7.2 mg per ml. The sedimentation coefficients were corrected to those in water at 20°.

Sucrose Gradient Centrifugation

H-RNA was fractionated by using a linear (5 to 20 %) sucrose gradient. The gradient was prepared in the phosphate buffer (0.02 M, pH 7.0). The RNA sample was layered on the sucrose

gradient and centrifuged for 40 hours at 24,000 rpm at 4°in a Hitachi Model 55PA ultracentrifuge with the RPS 25A rotor. After the centrifugation, the solution was fractionated from the bottom of the tube into 40 to 50 fractions. The absorbancy at 260 mμ of each fraction was measured. RNA preparations were obtained from the fractions as described above.

Further Fractionation of the Postribosomal Supernatant

The postribosomal supernatant (S-100) was centrifuged for 2 hours at 198,000 x g in a No. 50 rotor of a Spinco Model L ultracentrifuge. The upper 4/5 layer of the supernatant solution was aspirated (S-200). The lower 1/5 layer was decanted (S-200 B). The pellet was suspended in 0.05 M Tris-HCl (pH 7.8) containing 0.001 M magnesium acetate. The nucleic acid was extracted from each fraction by the phenol method.

Protein was determined according to the method of Lowry et al.[52]. RNA was estimated by measuring the optical density at 260 mμ.

3. RESULTS

Amounts of H-RNA in Cotyledons

TABLE XI shows the changes in the amounts of H-RNA in the cotyledons of 0 day- to 6 day-old seedlings. With the progress of germination, the amounts of H-RNA decreased but the contents of tRNA were almost constant. In the hypocotyls, tRNA was a main component of the soluble RNA fraction (S-100 RNA). In contrast, S-100 RNA from resting- and working-cotyledons contained much amount of H-RNA, and especially about 80 % of S-100 RNA from the resting-cotyledons were consisted of H-RNA.

TABLE XI. CHANGES OF THE AMOUNT OF H-RNA IN THE COTYLEDONS DURING GERMINATION OF SOYBEAN

Sources	S-100 RNA (mg per 100 g tissues)	H-RNA
0-coty	120	94
2-coty	64	43
4-coty	68	43
6-coty	61	37
3-hypo	43	7

Soybean seeds were germinated in vermiculite at 25° in the dark. Cotyledons (or hypocotyls) were separated from the seedlings at different times as indicated in the Table. RNAs were extracted, fractionated and estimated as described in MATERIALS AND METHODS.

TABLE XII. CHANGES OF THE AMOUNTS OF H-RNA IN THE COTYLEDONS DURING THE RIPENING PROCESS OF SOYBEAN KERNELS

Sources (weeks after flowering)	S-100 RNA (μg per one grain)	H-RNA
3	59	30
4	208	141
5	306	222
12 (at harvest)	852	723
	(137)	(116)

The kernels of soybean in the maturating stage were picked up from the field at different times. RNAs were extracted, fractionated and estimated as described in MATERIALS AND METHODS. The values in the parenthesis show the amounts of RNA as mg per 100 g tissues.

On the other hand, the amounts of H-RNA in the cotyledons in the developing soybean kernels were estimated by sampling at different times after flowering (TABLE XII). The amount of H-RNA increased much in the kernels with the progress of ripening, and the increase was parallel with that of protein content and of the

size of the kernels. The amount of tRNA increased gradually during ripening, but rRNA was almost constant through the ripening process (not presented). These results show that H-RNA found in the cotyledons of soybean seeds seems to be brought over from matured kernels.

Messenger Ability of H-RNA

The effect of various concentration of Mg^{2+} ions on the incorporation of amino acids into protein using E. coli S-30 system with and without H-RNA is shown in TABLE XIII. In CHAPTER IV, it was observed that H-RNA preparation stimulated leucine-^{14}C incorporation in the soybean system in the presence of 5 mM Mg^{2+} but not in 10 mM Mg^{2+}. The incorporation of amino acids was

TABLE XIII. EFFECT OF Mg^{2+} CONCENTRATION ON THE INCORPORATION OF AMINO ACIDS IN THE E. COLI S-30 SYSTEM

Concentration of Mg^{2+} (M)	Leucine-^{14}C incorporated H-RNA	
	without	with
	cpm per mg protein	
0.005	1710	1710
0.010	1180	17,110
0.015	1110	10,100

The amino acid incorporating system described in MATERIALS AND METHODS was used except that the concentrations of Mg^{2+} were varied. The amounts of H-RNA added was 500 μg.

observed in the E. coli system without adding H-RNA, and the incorporation was decreased in higher concentration of Mg^{2+}. In the presence of H-RNA, 10 mM Mg^{2+} proved to be optimum for amino acid incorporation, while the stimulating effect on amino acid incorporation by adding H-RNA was not observed in 5 mM of Mg^{2+}. The effects of various RNA preparations on amino acid incorpora-

tion in the E. coli S-30 system were therefore investigated in the presence of 10 mM of Mg^{2+} (TABLE XIV). The amino acid incorporation was enhanced by the addition of H-RNA preparations. H-RNA or rRNA from 3-hypo also showed the stimulating effect on amino acid incorporation. However, H-RNA from 0-coty was most effective. Transfer RNAs from yeast and soybean seedlings had no stimulating effect on amino acid incorporation.

TABLE XIV. EFFECTS OF RNA ON THE INCORPORATION OF AMINO ACIDS IN THE E. COLI S-30 SYSTEM

RNAs	leucine-^{14}C incorporated
	cpm per mg protein
none	1180
0-coty S-100 RNA	8990
" H-RNA	17,110
" H-RNA*	12,300
" tRNA	1290
3-hypo H-RNA	7150
" tRNA	1150
Yeast tRNA	1446
3-hypo rRNA	12,660
" rRNA	8720

The amino acid incorporating system described in MATERIALS AND METHODS was used. The amounts of RNAs used were 500 µg. In an asterisk (*), RNAs used were 250 µg.

The stimulation of amino acid incorporation was nearly proportional to the amount of H-RNA added, up to 500 µg RNA per tube (Fig. 10). It has been observed that the rate of incorporation was rapid at first and levels off after 20 minutes in the soybean seedlings system. In this experiment, leucine-^{14}C was incorporated continuously up to 100 minutes in the presence of H-RNA (500 µg). The incorporation of amino acids was decreased

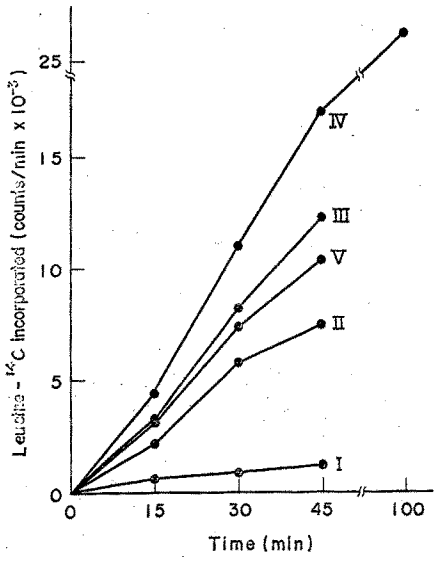

Fig. 10. Effect of H-RNA on the incorporation of leucine-^{14}C in the E. coli S-30 system.

The amino acid incorporating system is described in MATERIALS AND METHODS. The amount of H-RNA added was I, none; II, 125 µg; III, 250 µg; IV, 500 µg, respectively. V, IV minus amino acids.

by omitting of amino acid mixture.

Ultracentrifugal Analysis and Sucrose Gradient Fractionation of H-RNA

The sedimentation property of H-RNA from O-coty was studied in the Spinco E ultracentrifuge using schlieren optics. Sedimentation patterns are shown in Fig. 11. It was observed that H-RNA consisted of two major components, 18s and 9s ($S_{20,\ w}$). These components were then separated by sucrose gradient centrifugation described in MATERIALS AND METHODS (Fig. 12-A). Each component of H-RNA was not separated well, but two major peaks were clearly recognized and the patterns were reproducible. Each peak was collected, and the centrifugation was repeated in

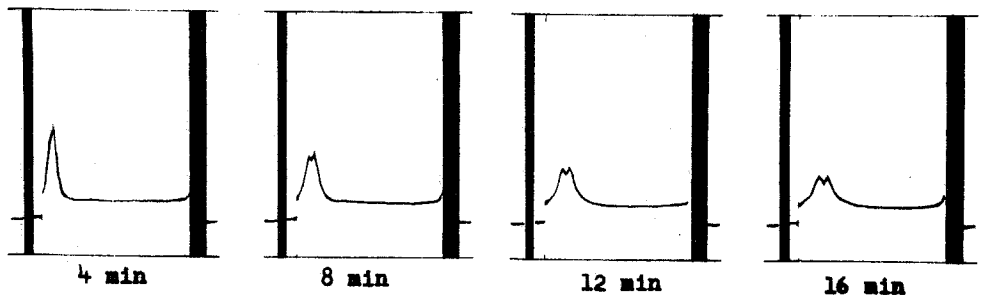

Fig. 11. Patterns of H-RNA preparation by the ultracentrifugal analysis.

Concentration of RNA was 7.2 mg/ml in 0.2 M KCl, 0.01 M phosphate buffer, pH 7.0. The centrifugation was carried out at 17.5°. Pictures were taken at 4 minutes intervals after reaching a speed of 56,100 rpm.

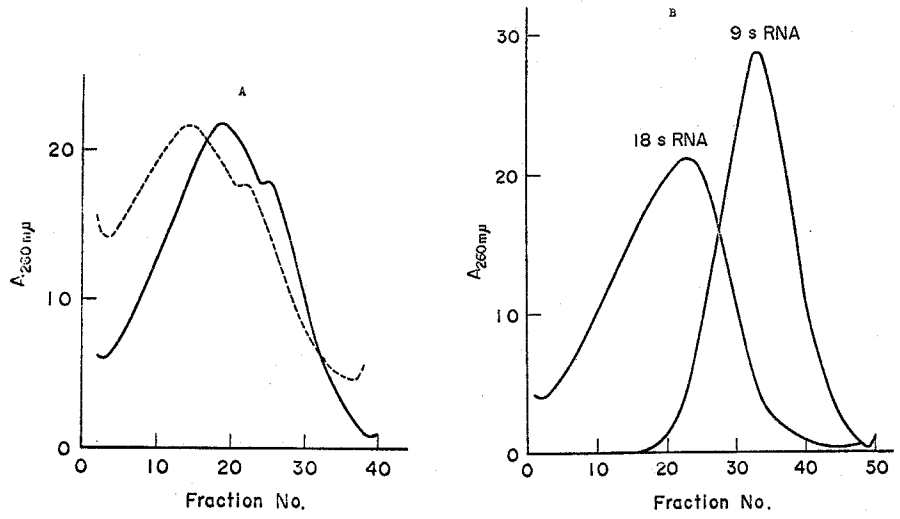

Fig. 12. Fractionation of H-RNA by the sucrose gradient centrifugation.

A— H-RNA was centrifuged and fractionated as described in MATERIALS AND METHODS. Dotted line shows the pattern of the different run of the same sample. B— Each peak shown in A was collected, dialyzed and precipitated by ethanol, and RNA preparations were obtained as described in MATERIALS AND METHODS. Each RNA preparation was centrifuged in the same sucrose gradient as in A.

the same sucrose gradient. Slower sedimenting component (9s RNA) showed a sharp peak and may not be contaminated with the faster sedimenting component (18s RNA) (Fig. 12-B). While the centrifugal pattern of the faster sedimenting component showed a broad peak and this fact may suggest that this component may be more heterogeneous than 9s RNA, but it looks clear that the faster sedimenting component might not be contaminated with 9s RNA. RNA of each peak was obtained as described in MATERIALS AND METHODS.

Messenger Ability of Each Component of H-RNA

Messenger abilities of two major components of H-RNA fractionated by the sucrose gradient centrifugation were assayed with the E. coli S-30 system (Fig. 13). The results show that both

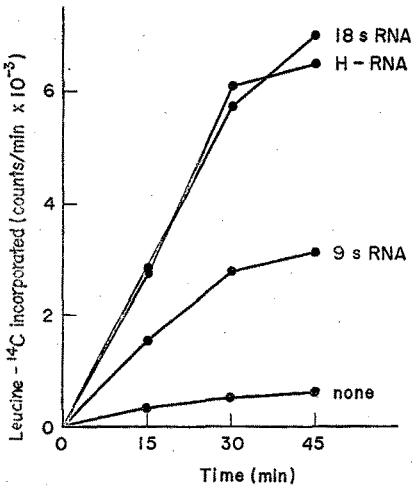

Fig. 13. Stimulation for the incorporation of leucine-^{14}C by the components of H-RNA fractionated by the sucrose gradient centrifugation.

The amino acid incorporating system described in MATERIALS AND METHODS was used. The amounts of H-RNA, 18s and 9s RNA were each 250 μg.

faster (18s) and slower (9s) sedimenting components have template

activities. The former stimulated the incorporation of amino acids as same as the original H-RNA, and the activity was about twice as much as the latter.

Further Fractionation of the Supernatant (S-100)

The supernatant (S-100) was further centrifuged for 2 hours at 198,000 x g to know whether these messenger-like RNA were existed as a free state or some other complexes, i.e. particulate components. As shown in TABLE XV, H-RNA was observed only a small amount in the supernatant from the centrifugation at 198,000 x g, while all of tRNAs were contained in the supernatant. It was observed that H-RNA was contained mainly in the precipitate fraction obtained from the centrifugation at 198,000 x g for 2 hours. These results suggest that H-RNA is existed as a particulate component.

TABLE XV. FURTHER FRACTIONATION OF S-100 BY ULTRACENTRIFUGATION

Fractions	Total amounts (mg/100 g tissues)	
	H-RNA	tRNA
S-100	139	31
S-200	13	31

Fractions	Amounts of RNA (OD_{260})
S-100	3.378
S-200	0.338
S-200 B	0.370
P-200	2.180

The soluble fraction (S-100) was centrifuged for 2 hours at 198,000 x g, and RNA was extracted from the resultant fractions as described in MATERIALS AND METHODS. In the lower column, the values were shown as a total absorbance per tube.

4. DISCUSSION

The present study demonstrates some characteristics of RNA preparations from soybean seeds having template activities. With the progress of maturation of kernels, the amount of H-RNA increased, and this H-RNA was kept stable in the seeds. These phenomena may be explained by the following reasons: (i) no cell divisions occured in the cotyledons after 15 days from flowering[67], (ii) the stabilization of mRNA occurs at a time, for example, during the maturation of the reticulocyte or the lens fiber cell, and therefore, the stabilization of mRNA appears to be associated with the loss of mitotic activity[68], and (iii) nuclease activities (PDase, RNase) in the cotyledons of seeds are very low compared with those in the hypocotyls (in CHAPTER II). However, it has been reported that an RNA fraction from dry seed of peanut, analogous to mRNA isolated from imbibed seed, did not stimulate the incorporation of amino acid into protein[66], while the existence of long-lived messenger RNA in plants has been suggested on the basis of experiments with actinomycin D by Morton and Raison[10] and Dure and Waters[69]. It has been also suggested that a long-lived messenger RNA unassociated with ribosomes was contained in the dormant seed of Pisum arvense[70].

As shown in the RESULTS, H-RNA preparation with template activities was analyzed by Spinco E ultracentrifugation, and two peaks with sedimentation values of 9s and 18s were observed. Both components fractionated by sucrose gradient centrifugation showed template activities in the E. coli S-30 system. In general, the most active material for stimulating amino acid incorporation is found associated with the 18s RNA obtained from nuc-

leus and cytoplasm, but the RNA species involved in the transfer of the genetic information in the 18s RNA are not clearly decided [65]. This RNA was seemed to be a mixture of mRNA and rRNA molecules considering from the nucleotide composition of 18s RNA[65]. It is also likely that H-RNA from soybean seeds may be a mixture of mRNA and other RNA molecules. Some RNAs, that is, nascent rRNA[71-73] and rRNA[74] whose secondary structures were disrupted have been reported to show template activities. Therefore, all the high template activities of H-RNA may not always be attributed to true messenger RNAs. On the other hand, 9s RNA from soybeans also possessed the template activities as shown in the RESULTS. This RNA seems not to be a degradative products of larger RNA molecules during isolation procedure since the pattern of the sucrose gradient analysis showed a sharp peak. Thus, whether 18s RNA and 9s RNA are the messenger RNA or mixture of some other RNA species has not yet been decided, but it may be possible to be decided from the data of the analysis of the base composition, of the hybridization or of the product directed by these RNAs.

Recently, it was reported that mRNAs were obtained from cerebral nuclei[75] and from germinating peanut cotyledons[66]. It was also reported that the RNA of the smooth membranes of rat liver contained the unusual RNA. The electrophoretic mobility of this RNA on polyacrylamide-gel was reported to be found between 18s and 5s RNA which are the constituents of rRNA, respectively. And this RNA showed higher template activity than rRNA[76].

Messenger RNAs have been derived from cell homogenates, polysomes[64], 40s ribonucleoprotein particles[77-79] and nucleus

(or nuclear ribonucleoproteins)[63]. However, the H-RNA was contained in the soluble fraction (supernatant of 100,000 x g, 2 hours centrifugation) of soybean seeds. When the soluble fraction was centrifuged for 2 hours at 198,000 x g, H-RNA was found in the precipitate. These results show that H-RNA does not exist as free state but may existed as some particulate component such as ribonucleoproteins.

CHAPTER VI

RIBONUCLEIC ACIDS THAT HAVE TEMPLATE ACTIVITIES FROM PARTICULATE COMPONENTS OF SOYBEAN SEEDS[e)]

1. INTRODUCTION

Occurrence of high molecular weight RNAs that have template activities in the soluble fraction of soybean seed (CHAPTER IV) and some characteristics of the RNA (CHAPTER V) have been described. These results showed that this RNA was consisted of two major components, 9s and 18s, which had template activities in the E. coli S-30 system. When the soluble fraction of soybean seeds was further centrifuged at 198,000 x g for 3 hours, most of the RNA was found in the precipitate (CHAPTER V). Existence of high molecular weight RNAs in the particulate components is presented in this hapter.

2. MATERIALS AND METHODS

Preparation of Subcellular Fractions

Soybean seeds soaked overnight in water at 4° and cotyledons which were detached from germs were homogenized in a mortar with 0.05 M Tris-HCl (pH 7.8) containing 0.25 M sucrose, 0.001 M magnesium acetate. The homogenate was squeezed through gauze and centrifuged for 30 minutes at 10,000 x g. The supernatant was centrifuged for 2 hours at 100,000 x g. The upper layer of the supernatant, about 4/5 part, was drawn out by pipetting (S-100). S-100 was treated with 0.5 % sodium deoxycholate and centrifuged for 3 hours at 198,000 x g. The upper layer, about 4/5 part of the supernatant was drawn out by pipetting (S-200). The pellet

was suspended in 0.05 M Tris-HCl (pH 7.8) containing 0.0001 M magnesium acetate (P-200). All operations were carried out below 4°.

Measurement of Template Activity

Amino acid incorporating system which was used corresponded to the S-30 fraction of Nirenberg and Matthaei[3] from E. coli (strain K-12). Aliquots of S-30 were incubated in a total volume of 0.5 ml, containing 25 μmoles Tris-HCl (pH 7.8), 25 μmoles potassium chloride, 5 μmoles magnesium acetate, 0.5 μmole ATP, 5 μmoles creatine phosphate, 25 μg creatine kinase, 0.125 μmole GTP, 5 μmoles 2-mercaptoethanol, 10 mμmoles each 19 amino acids, 2.22 mμmoles leucine-U-^{14}C (198 μc/μmole) and RNA preparation. Determination of leucine-^{14}C incorporated was measured by the method described in CHAPTER V.

Sucrose Gradient Centrifugation

P-200 was fractionated by 15-30 % (w/v) linear sucrose gradient containing 0.01 M Tris-HCl (pH 7.8) and 0.0001 M magnesium acetate. The sample was layered on 28 ml of sucrose gradient and centrifuged for 13 hours at 23,000 rpm at 4°. RNA was fractionated by 5-20 % (w/v) linear sucrose gradient containing 0.02 M phosphate buffer (pH 7.0). The sample was layered on 28 ml of sucrose gradient and centrifuged for 40 hours at 24,000 rpm at 4°. After centrifugation, the gradients were fractionated through a flow cell connected to an continuous recording system in a density gradient fractionator (ISCO Co.). In the case of P-200, the corresponding fractions were pooled from several runs, concentrated with Sephadex G-25 and dialyzed against 0.01 M Tris-HCl (pH 7.8) containing 0.0001 M magnesium acetate. Centrifuga-

tion was carried out in a Hitachi Model 55PA preparative ultracentrifuge with a RPS 25A rotor.

S-100 was fractionated into high (H-RNA) and low (tRNA) molecular weight with a Sephadex G-200 column as shown in Fig. 14.

The amount of protein was determined by Lowry method[52].

Methods of preparation and estimation of RNA were the same as those described in CHAPTER V.

3. RESULTS

Fig. 14 shows the chromatographic patterns of RNA preparations from the supernatants of 100,000 x g (S-100) and 198,000 x g (S-200) centrifugation. H-RNA which existed in S-100 was not

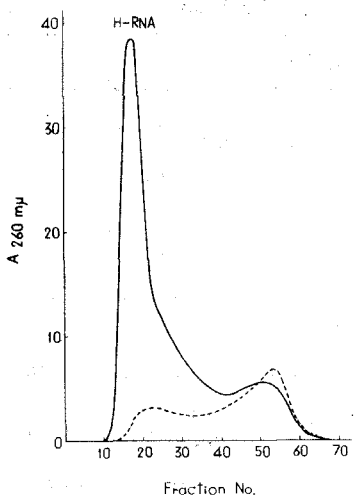

Fig. 14. Chromatographic analysis of RNAs on Sephadex G-200 column.

S-100 (or S-200) RNA solution was applied to a column (2.5 x 50 cm) and eluted with 2 % potassium acetate adjusted pH at 6. Each 3 ml fraction was collected, and the absorbance at 260 mμ was measured. ———, S-100 RNA; – – –, S-200 RNA.

found in S-200 (The peak eluted in a void volume corresponds to H-RNA as described in CHAPTERs IV and V).

The precipitate from the centrifugation at 198,000 x g (P-200) was centrifuged in a sucrose gradient (Fig. 15-A). And particulate components (peak II) were found in the P-200. As

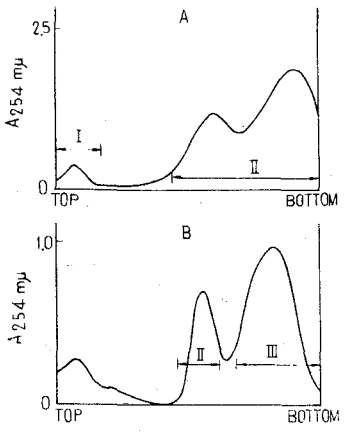

Fig. 15. Sucrose gradient analysis of P-200.

A, 0.5 ml of P-200 (30 OD_{260} units) was layered on a sucrose gradient, centrifuged and fractionated as described in MATERIALS AND METHODS. B, 0.5 ml of lower concentration of P-200 (8 OD_{260} units) was used. Peaks I and II (in A) and peaks II and III (in B) were collected separately and pooled from several runs, and these were used for the experiment shown in TABLE XVI and Fig. 16, respectively.

shown in TABLE XVI, almost all of the RNA was found in peak II. These experiments suggest that H-RNA is contained in the particulate components. As shown in Fig. 15-B, when the lower concentration of P-200 was used as a sample, each component was clearly resolved. The size of these particulate components were measured by analytical ultracentrifuge as about 37s and 59s, respectively (in 0.01 M Tris-HCl, pH 7.8 and 0.0001 M magnesium acetate).

TABLE XVI. DISTRIBUTION OF RNA IN P-200

Fractions	Total amount of RNA (absorbance units at 260 mμ)		
	Experiments		
	1	2	3
P-200	200	267	312
I	16	5	7
II	134	181	215

RNAs prepared from each particulate component which was prepared by the sucrose gradient centrifugation (as shown in Fig.

15-B) were analyzed by sucrose gradient centrifugation (Fig. 16). The RNA preparation from 59s particles contained large RNA component of H-RNA (18s RNA) and some other smaller components, and that from 37s particles contained small RNA component of H-RNA (9s RNA) and some other smaller components but not 18s RNA. These patterns were reproducible (Fig. 16). As shown in TABLE XVII,

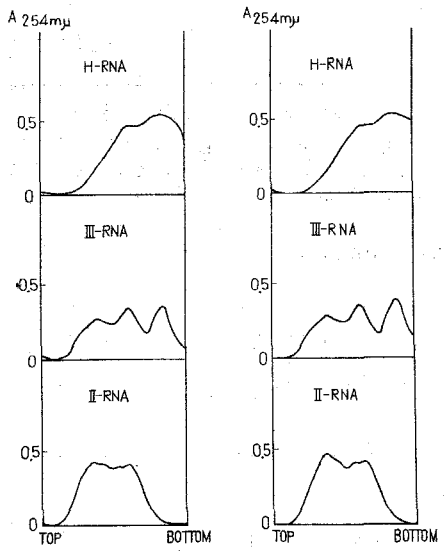

Fig. 16. Sucrose density gradient analysis of RNAs obtained from the particulate components.

H-RNA was prepared as described in the text. II- and III-RNA were prepared from peaks II and III obtained as shown in Fig. 15-B. 0.3 ml of each RNA solution (H-RNA, 17 OD_{260} units; II- and III-RNA, 12 OD_{260} units) was layered on the gradients and centrifuged. The analysis was carried out by passing through a recording system (ISCO).

these RNA preparations, II- and III-RNA prepared from the particulate components, showed stimulative effects for the amino acid incorporation in E. coli S-30 system, and III-RNA was more stimulative than II-RNA. However, the original particulate components were almost inactive for the stimulation of amino acid incorporation in E. coli and soybean seedling systems.

TABLE XVII. EFFECTS OF RNA PREPARATIONS ON THE AMINO ACID INCORPORATING SYSTEM OF E. COLI

Additions	Leucine-^{14}C incorporated (cpm per tube)
none	650
H-RNA	1554
H-RNA*	2424
II-RNA	1043
III-RNA	1880

The reaction mixture described in MATERIALS AND METHODS containing each 60 μg of RNA preparation and 0.42 mg protein of S-30 was incubated for 20 minutes at 37°. RNA was prepared as shown in the text and Fig. 16.
*, 120 μg RNA was added.

As described in MATERIALS AND METHODS, the precipitate obtained from the centrifugation at 198,000 x g was suspended in 10^{-4} M magnesium acetate buffer. The increase of Mg^{2+} (even to

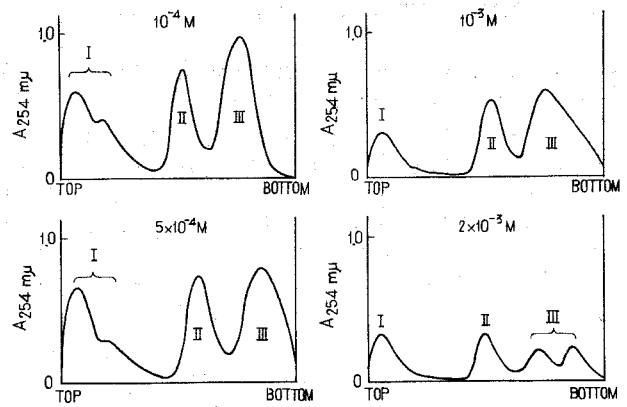

Fig. 17. Sucrose density gradient analysis of the particulate components at various concentrations of Mg^{2+}.

P-200 was prepared as described in MATERIALS AND METHODS and was suspended in the buffer contained Mg^{2+} at designated concentration. Operation was the same as shown in Fig. 15. 0.5 ml of P-200 (13 OD_{260} units) was used. Peaks I, II and III were collected separately and used for the experiment shown in TABLE XVIII.

10^{-3} M) caused turbidity. As shown in Fig. 17, a half of the component of peak I was precipitated at 10^{-3} M Mg^{2+}, but the particulate components were not affected. The occurrence of the turbidity in suspending at 10^{-3} M Mg^{2+} may be due to the component of peak I. When the concentration of Mg^{2+} was increased to 2×10^{-3} M, about a half of each particulate component was precipitated, and peak III was more precipitable than peak II (Fig. 17 and TABLE XVIII).

TABLE XVIII. EFFECTS OF Mg^{2+} CONCENTRATION ON THE PARTICULATE COMPONENTS

Peaks	Total absorbance of each peak at 260 mμ			
	Concentration of Mg^{2+}			
	10^{-4}	5×10^{-4}	10^{-3}	2×10^{-3}
I	4.64	4.25	2.19	1.80
II	3.05	3.05	2.80	1.53
III	5.02	4.52	4.77	2.01
Total (%)	100	93	77	42

Peaks I, II and III were obtained from each gradient containing various concentrations of Mg^{2+} as shown in Fig. 17, and the absorbance at 260 mμ was measured.

4. DISCUSSION

The results presented above showed that the RNAs which have template activities existed as particulate components, 37s and 59s particles. As shown in Fig. 16, the large RNA component (18s RNA) seemed to be contained only in 59s particles, and the smaller component (9s RNA) to be contained in both particles. However, it was observed that the RNAs in the particulate form were not stable during storage for a few days at 4°considering from

the result of stability experiment (the data were not presented). From this fact, it seems likely that the smaller RNA components of 59s particles may be a partially degraded product of the large RNA components. However, 9s RNA from 59s particles showed a sharp peak, therefore, they may not be regarded as the degradation product of 18s RNA. On the other hand, 18s RNA was not found in RNA preparation of 37s particles. And 37s particles contain 9s RNA and the smaller RNA components which seem to be degraded products of 9s RNA.

The difference in stimulative effects of RNAs from particulate components on the amino acid incorporation in S-30 system (TABLE XVII) was in good agreement with the result (described in CHAPTER V), in which 9s and 18s RNA were prepared from H-RNA by sucrose density gradient centrifugation, and 18s RNA was about 2 times more stimulative than 9s RNA (CHAPTER V). From these results, it may be concluded that the large and small RNA components obtained from each particulate component correspond to 18s RNA and 9s RNA, respectively.

The existence of the particulate components containing RNAs which have template activities in cotyledons of soybean seeds may be interesting considering from the current studies on subribosomal particles containing messenger RNA found in nucleus or cytoplasm[80-90]. Those particles have been reported to be 30s, 40s or 45s, and those may differ from the particulate components in soybeans which are 37s and 59s. However, as shown in Fig. 17 and TABLE XVIII, there were little changes in size and the amount of the particulate components at 10^{-4} to 10^{-3} M and both particulate components became precipitable at 2×10^{-3} M. These facts

may suggest that the particulate components are different from the subunits of ribosomes. And also, the particulate components were different from ribosomes in absorption pattern. OD ratios (260/280) of the particulate components and ribosomes from soybean seedlings are 1.67-1.72 and 1.90, respectively. Recently, it has been shown that the size of the particles containing messenger RNAs are not uniform, and beside such particles, there are some more different sizes of particles[87-89, 91, 92]. Those facts and results described above may suggest that the particulate components found in cotyledons of soybean seeds are similar to those subribosomal particles containing messenger RNA found in nucleus or cytoplasm described above.

SUMMARY

The auther has attempted to elucidate the protein synthesizing system of soybean seeds and seedlings *in vitro*, and the results obtained are summarized as follows.

In CHAPTER II, ribosomes from cotyledons of soybean seeds soaked overnight in water (resting-cotyledon ribosomes) and hypocotyls (hypocotyl ribosomes) of 5 day-old seedlings were prepared. These ribosomes mainly consisted of 80s particles. However, hypocotyls contained 115s particles in a small amount and working-cotyledons contained 115 and 150s particles which may correspond to the polymers of ribosomes or polysomes. The abundant 80s ribosomes were fractionated by the sucrose density gradient centrifugation, and enzyme activities in the fractionated ribosomes were estimated. RNase, PDase, acid phosphatase, 5′-nucleotidase, XTPase, peroxidase and β-glucosidase were found in the ribosomes. The enzyme activities were lower in the resting-cotyledon ribosomes and higher in the hypocotyl ribosomes. Working-cotyledon ribosomes showed the middle of them. All these enzymes were also found in the cytoplasmic solution (supernatant) of cotyledon and hypocotyl cells. However, RNase, PDase, 5′-nucleotidase and peroxidase were concentrated in ribosomes, and the specific activities of 5′-nucleotidase and β-glucosidase were increased by washing the ribosomes. The status of the enzymes found in the ribosomes was discussed.

In CHAPTER III, ribosomes were prepared from the cotyledons of soybean seeds soaked overnight in water (resting-cotyledon ribosomes) and from the cotyledons and the hypocotyls of seedlings (working-cotyledon and hypocotyl ribosomes), respectively.

As to the amino acid incorporating activities, resting-cotyledon ribosomes were inactive, and working-cotyledon and hypocotyl ribosomes were active. The supernatant from the homogenate of resting-cotyledons contained sRNA and the enzyme systems necessary for the amino acid incorporation. Resting-cotyledon ribosomes were supposed to be deficient in some other factors. Polysome fractions separated from working-cotyledon and hypocotyl ribosomes by the sucrose gradient centrifugation showed higher specific activities than monosome fraction from the same source. When the cotyledons and the hypocotyls from the seedlings were immersed in ^{32}P-orthophosphate solution, ^{32}P was incorporated rapidly in polysome fractions of these active tissues (mRNA formation). However, the incorporation was not observed remarkably in the ribosomes from resting cotyledons. These results may suggest that the differences in the incorporating activities of the ribosome preparations due to the occurrence and the amount of polysomes in the preparations. Therefore, the amino acid incorporating system in the resting cotyledons is assumed to be deficient in polysomes. Ribosomes in the resting cotyledons may be inactive to form polysomes. The protein synthesizing systems in the cotyledons is activated as the seeds germinate.

In CHAPTER IV, the existence of RNAs having template activities in the 100,000 x g supernatant fraction from soybean seeds was observed.

In CHAPTER V, the RNAs having template activities were extracted from soluble fraction of the cotyledons of soybean seeds. These were consisted of two major components, 9s and 18s (High molecular weight RNA, H-RNA). Both components have template

activities in the E. coli S-30 system. H-RNA was found in the precipitate fraction when the so-called soluble fraction was centrifuged for 2 hours at 198,000 x g. H-RNA increased remarkably in kernels during ripening process and seems to be preserved in the seeds.

In CHAPTER IV, ribonucleic acids that have template activities were obtained from particulate components prepared from the post ribosomal supernatant of soybean seeds. These RNAs were 9s and 18s in size, and these corresponded to those of H-RNA prepared from the supernatant of 100,000 x g centrifugation. The sizes of the particulate components were 37s and 59s, respectively. Larger particles contained 18s and 9s RNA, and smaller particles contained 9s RNA, but not 18s RNA. Those particulate components differed in absorption pattern and in the behaviour on sucrose gradient centrifugation depending on the concentration of Mg^{2+} from the subunits of ribosomes.

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REFFERENCES

1) P. Berg, Ann. Rev. Biochem., 30, 293 (1961).

2) M. V. Simpson, ibid., 31, 333 (1962).

3) R. Schweet and R. Heintz, ibid., 35 Part II, 723 (1966).

4) G. C. Webster, Arch. Biochem. Biophys., 85, 159 (1959).

5) I. D. Raacke, Biochim. Biophys. Acta, 34, 1 (1959).

6) J. T. Lett, W. N. Takahashi and M. Birnstiel, ibid., 76, 105 (1963).

7) R. Rabson and G. D. Novelli, Proc. Natl. Acad. Sci. U. S., 46, 484 (1960).

8) R. J. Mans and G. D. Novelli, Biochim. Biophys, Acta, 80, 127 (1964).

9) R. J. Mans, C. M. Purcell and G. D. Novelli, J. Biol. Chem., 239, 1762 (1964).

10) R. K. Morton and J. K. Raison, Biochem. J., 91, 528 (1964).

11) A. Marcus and J. Feeley, Proc. Natl. Acad. Sci. U. S., 51, 1075 (1964).

12) A. Marcus and J. Feeley, J. Biol. Chem., 240, 1675 (1965).

13) A. Marcus, J. Feeley and T. Volcani, Plant Physiol., 41, 1167 (1966).

14) B. Parisi and O. Ciferri, Biochemistry, 5, 1638 (1966).

15) J. L. Young and J. E. Varner, Arch. Biochem. Biophys., 84, 71 (1959).

16) J. E. Varner and G. R. Chandra, Proc. Natl. Acad. Sci. U. S., 52, 100 (1964).

17) M. L. Peterman, "The Physical and Chemical Properties of Ribosome", Elsevier Publish Co., 1964, p.164.

18) M. Tal and D. Elson, Biochim. Biophys. Acta, 72, 439 (1963).

19) M. Tal and D. Elson, ibid., 76, 40 (1963).

20) Z. Fogel and D. Elson, ibid., 80, 601 (1964).

21) J. P. Waller, J. Mol. Biol., 10, 319 (1964).

22) R. J. Britten and R. B. Roberts, Science, 131, 32 (1960).

23) "Methods in Enzymology", Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1957, p. 447.

24) ibid., Vol. III, 1957, p. 87.

25) S. Matsushita, Mem. Res. Inst. Food Sci., Kyoto Univ., 17, 23 (1959).

26) S. Matsushita, F. Ibuki, T. Mori and T. Hata, Agr. Biol. Chem., 29, 436 (1965).

27) R. J. L. Allen, Biochem. J., 34, 858 (1940).

28) T. Hosoya, J. Biochem. (Tokyo), 47, 794 (1960).

29) H. Kihara, A. S. L. Hu and H. O. Halvorson, Proc. Natl. Acad. Sci. U. S., 47, 489 (1961).

30) "Methods in Enzymology", Vol. V, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1962, p. 601.

31) ibid., Vol. I, 1955, p. 449.

32) ibid., Vol. V, 1962, p. 703.

33) ibid., Vol. I, 1955, p. 301.

34) ibid., Vol. III, 1957, p.201.

35) ibid., Vol. I, 1955, p. 149.

36) A. T. Matheson and C. S. Tsai, Canad. J. Biochem., 43, 323 (1965).

37) A. Rich, J. R. Warner and H. M. Goodman, Cold Spring Harbor Symp. Quant. Biol., 28, 269 (1963).

38) J. R. Warner, A. Rich and C. E. Hall, Science, 138, 1399 (1962).

39) M. F. Clark, R. E. F. Matthews and R. K. Ralph, Biochim. Biophys. Acta, 91, 289 (1964).

40) S. T. Bayley, J. Mol. Biol., 8, 231 (1964).

41) M. Takanami, Biochim. Biophys. Acta, 39, 318 (1960).

42) I. D. Raacke and J. Fiala, Proc. Natl. Acad. Sci. U. S., 51, 323 (1964).

43) I. D. Raacke, Federation Proc., 22, 348 (1963).

44) H. C. Neu and L. A. Heppel, Biochem. Biophys. Res. Comm., 14, 109 (1964).

45) H. C. Neu and L. A. Heppel, ibid., 17, 215 (1964).

46) M. Balamy and B. L. Horecker, ibid., 5, 104 (1961).

47) M. F. Singer and P. Leder, Ann. Rev. Biochem., 35, Part I, 195 (1966).

48) U. E. Loening, Biochem. J., 97, 125 (1965).

49) M. F. Clark, R. E. F. Mattews and R. K. Ralph, Biochem. Biophys. Res. Comm., 13, 505 (1963).

50) H. Chroboczek and J. H. Cherry, ibid., 20, 774 (1965).

51) K. S. Kirby, Biochem. J., 64, 405 (1956).

52) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951).

53) F. Lipmann, Proc. Natl. Acad. Sci. U. S., 44, 67 (1958).

54) R. Rendi and P. N. Campbell, Biochem. J., 72, 435 (1959).

55) J. R. Warner, P. M. Knopf and A. Rich, Proc. Natl. Acad. Sci. U. S., 49, 122 (1963).

56) R. Monier, M. L. Stephinson and P. C. Zamecnik, Biochim. Biophys. Acta, 43, 1 (1960).

57) P. Berg, F. H. Bergmann, E. J. Ofengand and M. Dieckmann, J. Biol. Chem., 236, 1726 (1961).

58) "Methods in Enzymology", Vol. VI, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y., 1963, p. 17.

59) E. Volkin and L. Astrachan, "The chemistry of Heredity", Johns Hopkins Press, Baltimore, 1957, p. 686.

60) K. S. Kirby, "Techniques in Protein Biosynthesis", Vol. I, Academic Press Inc., New York, N. Y., 1967, p.265.

61) "Progress in Nucleic Acid Research", Vol. I, ed. by J. N. Davidson and Waldo E. Cohn, Academic Press Inc., New York, N. Y., 1963, p. 135.

62) "Progress in Nucleic Acid Research and Molecular Biology", Vol. III, ed. by J. N. Davidson and Waldo E. Cohn, Academic Press Inc., New York, N. Y., 1964, p. 1.

63) ibid., Vol. VI, 1967, p. 259.

64) ibid., Vol. VII, 1967, p. 173.

65) ibid., Vol. VII, 1967, p. 195.

66) W. J. Jachymczyk and Joe H. Cherry, Biochim. Biophys. Acta, 157, 368 (1968).

67) A. G. Norman, "The Soybean", Academic Press, New York, N. Y., 1963, p. 104.

68) J. A. Stewart and J. Papaconstantinou, J. Mol. Biol., 29, 357 (1967).

69) L. Dure and L. Waters, Science, 147, 410 (1965).

70) G. R. Barker and M. Rieber, Biochem. J., 105, 1195 (1967).

71) E. Otaka, S. Osawa and A. Sibatani, Biochem. Biophys. Res. Comm., 15, 568 (1964).

72) D. Nakada, J. Mol. Biol., 12, 695 (1965).

73) A. Muto, ibid., 36, 1 (1968).

74) J. J. Holland, C. A. Buck and B. J. McCarthy, Biochemistry, 5, 358 (1966).

75) S. C. Bondy and Sidney Roberts, Biochem. J., 105, 1111 (1967).

76) H. W. S. King and W. Fitschen, Biochim. Biophys. Acta, 155, 32 (1968).

77) W. K. Joklik and Y. Becker, J. Mol. Biol., 13, 496 (1965).

78) E. C. Henshaw, M. Revel and H. H. Hiatt, ibid., 14, 241 (1965).

79) E. H. McConkey and J. W. Hopkins, ibid., 14, 257 (1965).

80) "Progress in Nucleic Acid Research and Molecular Biology", Vol. VII, ed. by J. N. Davidson and Waldo E. Cohn, Academic Press Inc., New York, N. Y., 1967, p. 231.

81) ibid., p. 283.

82) O. P. Samarina, A. A. Krichevskaya and G. P. Georgiev, Nature, 210, 1319 (1966).

83) J. T. Parsons and K. S. McCarty, J. Biol. Chem., 243, 5377 (1968).

84) K. Köhler and S. Arends, European J. Biochem., 5, 500 (1968).

85) Y. Moulé and J. Chauveau, J. Mol. Biol., 33, 465 (1968).

86) R. P. Perry and D. E. Kelley, ibid., 16, 255 (1966).

87) A. A. Infante and M. Nemer, ibid., 32, 543 (1968).

88) E. C. Henshaw, ibid., 36, 401 (1968).

89) F. C. Kafatos, Proc. Natl. Acad. Sci. U. S., 59, 1251 (1968).

90) P. F. Benson, Biochem. J., 110, 59p (1968).

91) O. P. Samarina, E. M. Lukanidin and G. P. Georgiev, Biochim. Biophys. Acta, 142, 561 (1967).

92) O. P. Samarina, E. M. Lukanidin, J. Molnar and G. P. Geogiev, J. Mol. Biol., 33, 251 (1968).

a) S. Matsushita, T. Mori and T. Hata, Plant and Cell Physiol., 7, 533 (1966).

b) S. Matsushita, T. Mori and T. Hata, Mem. Res. Inst. Food Sci., Kyoto Univ., 29, 1 (1968).

c) T. Mori, F. Ibuki, S. Matsushita and T. Hata, Arch. Biochem. Biophys., 124, 607 (1968).

d) T. Mori, F. Ibuki, S. Matsushita and T. Hata, Agr. Biol. Chem., 33, 1229 (1969).

e) T. Mori and S. Matsushita, Agr. Biol. Chem., in contribution.